

In re Patent Application of: Masaaki Kosaka et al.

Serial No. 10/069,290

Examiner: SEHARASEYON, JEGATHEESAN

Filed: February 25, 2002

Group Art Unit: 1647

For: EXPRESSION ENHANCER FOR HM

1.24 ANTIGEN



TRANSLATOR'S DECLARATION

Honorable Commissioner of Patents & Trademarks  
Washington, D.C. 20231

Sir:

I, Tsumoru Fukumoto, residing at c/o A. AOKI, ISHIDA & ASSOCIATES, Toranomom 37 Mori Bldg., 3-5-1, Toranomom Minato-ku, Tokyo 105-8423, Japan declare the following:

(1) That I know well both the Japanese and English languages;

(2) That I translated Japanese Patent Application No. 2000-038689, filed February 16, 2000, from the Japanese language to the English language;

(3) That the attached English translation is a true and correct translation of the aforesaid Japanese Patent Application No. 2000-038689 to the best of my knowledge and belief; and

(4) That all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such false statements may jeopardize the validity of the application or any patent issuing thereon.

July 26, 2005

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Date

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Translator Tsumoru Fukumoto

[NAME OF DOCUMENT] APPLICATION FOR PATENT

[REFERENCE NUMBER] 1003117

[DATE FILED] February 16, 2000

[DESTINATION] To Commissioner, Patent Office;  
Mr. Takahiko Kondo

[INTERNATIONAL PATENT CLASSIFICATION] A61K 38/18  
A61K 39/00

[TITLE OF THE INVENTION] Expression Enhancer for HM1.24  
Antigen

[NUMBER OF CLAIMS] 20

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[PRIOR APPLICATION FROM WHICH PRIORITY IS CLAIMED]

[Application Number]	11-236007
[Application Date]	August 23, 1999

[INDICATION OF FEES TO BE PAID]

[Registration Number for Prepayment]	036135
[Amount of Fee]	21000

[LIST OF ARTICLES TO BE SUBMITTED]

[Name of Article]	Specification	1
[Name of Article]	Drawing	1
[Name of Article]	Abstract	1
[Number of General Authorization]		9814920

[NEED FOR PROOF]

Yes



ingredient.

[Claim 11] A therapeutic agent for myeloma comprising, as an active ingredient:

- (1) IRF-2 protein, and
- (2) an antibody that specifically binds to a protein having the amino acid sequence as set forth in SEQ ID NO: 2, and that has a cytotoxic activity.

[Claim 12] The therapeutic agent according to claim 11 wherein said myeloma is multiple myeloma.

[Claim 13] The therapeutic agent according to claim 11 or 12 wherein said antibody is a monoclonal antibody.

[Claim 14] The therapeutic agent according to claim 13 wherein said antibody has a cytotoxic activity.

[Claim 15] The therapeutic agent according to claim 11 wherein said antibody is a chimeric antibody or a humanized antibody.

[Claim 16] The therapeutic agent according to claim 14 wherein said antibody is anti-HM1.24 antibody.

[Claim 17] The therapeutic agent according to claim 15 wherein said chimeric antibody or humanized antibody is chimeric anti-HM1.24 antibody or humanized anti-HM1.24 antibody.

[Claim 18] An enhancer for expression in myeloma cell of HM1.24 antigen, said enhancer comprising, as an active ingredient, a compound that enhances the expression of IRF-2 protein.

[Claim 19] An activating agent for the HM1.24 promoter, said agent comprising, as an active ingredient, a compound that enhances the expression of IRF-2 protein.

[Claim 20] A method of screening expression enhancers of HM1.24 antigen.

[DETAILED DESCRIPTION OF INVENTION]

[0001]

[TECHNICAL FIELD TO WHICH INVENTION BELONGS]

The present invention relates to the use of interferon- $\alpha$ , interferon- $\gamma$ , and IRF-2 protein as an expression enhancer

for HM1.24 antigen in myeloma.

[0002]

[CONVENTIONAL ART]

Myeloma, also termed as plasmacytoma and multiple myeloma, is a neoplastic disease characterized by the accumulation of monoclonal plasma cells in the bone marrow. Myeloma is a disease in which terminally differentiated B cells or plasma cells that produce and secrete immunoglobulins are monoclonally increased predominantly in the bone marrow, and thereby monoclonal immunoglobulins or their constituent light chains or heavy chains are detected in the blood of patients with this disease.

For the treatment of myeloma, chemotherapeutic agents have so far been used, but no effective therapeutic agents have been discovered that lead to complete remission of the disease and that prolong the survival period of patients with myeloma, and thereby the appearance of drugs that have therapeutic effects of myeloma have long been sought after.

[0003]

On the other hand, Goto, T. et al. have reported a monoclonal antibody (mouse anti-HM1.24 antibody) that was obtained by immunizing mice with human myeloma cells (Blood (1994) 84, 1922-1930). When anti-HM1.24 antibody was administered to a mouse transplanted with human myeloma cells, the antibody accumulated in tumor tissues in a specific manner (Masaaki Kosaka et al., Nippon Rinsho (Japan Clinical) (1995) 53, 627-635), suggesting that anti-HM1.24 antibody could be applied in the diagnosis of tumor localization by radioisotopic labeling, missile therapies such as radiotherapy, and the like.

[0004]

In the above Blood (1994) 84, 1922-1930, it has been described that anti-HM1.24 antibody has an in vitro cytotoxic activity on a human myeloma cell line RPMI8226. It has also been shown that chimeric anti-HM1.24 antibody that is mouse anti-HM1.24 antibody turned chimeric, and a humanized reshaped

anti-HM1.24 antibody specifically bind to myeloma cells and have a cytotoxic activity (Blood (1999) 93, 3922-3920).

[0005]

Thus, HM1.24 antigen has been highly expressed specifically on myeloma cells that are terminally differentiated B cells and, as the anti-HM1.24 antibody that recognizes this antigen exhibits a cell-killing activity in proportion to the number of HM1.24 molecules on the cell surface, an immunotherapy employing anti-HM1.24 antibody is thought to be an effective method of treating multiple myeloma. Thus, if the amount expressed of HM1.24 antigen, an antigen of anti-HM1.24 antibody, on the cell surface could be enhanced, the administration of a smaller amount of the antibody is expected to provide an equal cytotoxic activity thereby lowering side effects.

[0006]

On the other hand, interferon was discovered to be a substance that exhibits a suppressing activity of viral growth and is known to be classified into four groups of  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\omega$ , and to have a variety of biological activities (Pestka, S., et al., Ann. Rev. Biochem. (1987) 56, 727-777; Langer, J. A., et al., Immunology Today (1988) 9, 393-400). However, there were no reports on the fact that interferon- $\alpha$  and interferon- $\gamma$  had an effect of increasing the amount expressed of HM1.24 antigen in myeloma cells.

[0007]

On the other hand, interferon regulatory factor (IRF)-1 and -2 were identified as transcription regulatory factors of the IFN- $\beta$  gene. IRF-1 and -2 are known to bind to the same gene regulatory sequence: IRF-1 and IRF-2 act in an antagonistic manner in that IRF-1 acts as a transcription activation factor, whereas IRF-2 acts as a transcription suppressing factor. The NIH3T3 cells in which IRF-2 was highly expressed have been demonstrated to exhibit enhanced cell saturation density, colony formation in the

methycellulose gel, and a tumorigenic property in nude mice, and IRF-2 acts as an oncogene.

[0008]

On the other hand, recent advances in research have indicated that IRF-2 is required for the expression of histone H4 that acts to control the cell cycle. IRF-2 is also shown to increase the expression of vascular cell adhesion molecule-1 (VCAM-1) in muscle cells, and it is becoming increasingly clear that the acid region (182 to 218) is involved in the activation of VCAM-1. Based on this, it is known that IRF-2 not only acts as a transcription regulatory factor but as a transcription activation factor.

However, it was not known that IRF-2 protein binds to the promoter (HM1.24 promoter) of the HM1.24 antigen gene, and activates said promoter.

[0009]

[PROBLEMS TO BE SOLVED BY THE INVENTION]

Current methods of treating myeloma are, as mentioned above, not satisfactory yet and, accordingly, the appearance of epoch-making therapeutic drugs or methods that prolong the patient's survival are awaited. The treatment of myeloma with anti-HM1.24 antibody is likely to be an epoch-making therapeutic treatment and thus there is a need for methods of exhibiting the effect of anti-HM1.24 antibody more efficiently.

Thus, it is an object of the present invention to provide means to enhance the myeloma-suppressing effect of anti-HM1.24 antibody by increasing the amount expressed of HM1.24 antigen in myeloma cells.

[0010]

[MEANS FOR SOLVING THE PROBLEMS]

In order to provide such methods, the inventors of the present invention have carried out a search for drugs that enhance the expressed amount of HM1.24 antigen, and as a result, have found that interferon- $\alpha$  and interferon- $\gamma$  have the desired effects, and thereby have completed the present



invention.

Thus, the present invention provides an enhancer of expression in the myeloma cell of a protein (HM1.24 antigen) having the amino acid sequence as set forth in SEQ ID NO: 2, said enhancer comprising interferon- $\alpha$  or interferon- $\gamma$  as an active ingredient.

[0011]

The present invention also provides a therapeutic agent for myeloma, said agent comprising, as an active ingredient:

- (1) interferon- $\alpha$  or interferon- $\gamma$ , and
- (2) an antibody that specifically binds to a protein having the amino acid sequence as set forth in SEQ ID NO: 2, and that has a cytotoxic activity.

Typical of the above myeloma is multiple myeloma.

Said antibody is preferably a monoclonal antibody, a chimeric antibody or a humanized antibody, and preferably has a cytotoxic activity.

[0012]

The inventors of the present invention have carried out a search for activating agents of the HM1.24 promoter, and have found that IRF-2 protein has the desired activity, and thereby have completed the present invention.

Thus, the present invention provides an enhancer of expression in the myeloma cell of a protein (HM1.24 antigen) having the amino acid sequence as set forth in SEQ ID NO: 2, said enhancer comprising IRF-2 protein as an active ingredient.

The present invention also provides an activating agent of the HM1.24 promoter, said agent comprising IRF-2 protein as an active ingredient.

[0013]

The present invention also provides a therapeutic agent for myeloma comprising, as an active ingredient:

- (1) IRF-2 protein, and
- (2) an antibody that specifically binds to a protein having the amino acid sequence as set forth in SEQ ID NO: 2,

and that has a cytotoxic activity.

Typical of the above myeloma is multiple myeloma.

Said antibody is preferably a monoclonal antibody, a chimeric antibody or a humanized antibody, and preferably has a cytotoxic activity.

[0014]

The present invention also provides an enhancer of expression in the myeloma cell of HM1.24 antigen, said enhancer comprising, as an active ingredient, a compound that enhances the expression of IRF-2 protein.

The present invention also provides an activating agent of the HM1.24 promoter, said agent comprising a compound that enhances the expression of IRF-2 protein.

The present invention also provides a method of screening agents that enhance the expression of HM1.24 antigen.

[0015]

[EMBODIMENT FOR CARRYING OUT THE INVENTION]

Interferon- $\alpha$  and interferon- $\gamma$

Interferon- $\alpha$  and interferon- $\gamma$  for use in the present invention may be mutants as long as they have an activity of increasing the amount expressed of HM1.24 antigen. In order to determine the amount expressed of HM1.24 antigen, as described in Examples, myeloma cells are harvested from a myeloma cell line or a patient with myeloma and then subjected to flow cytometry for detection. As mutants, they may be interferon- $\alpha$  and interferon- $\gamma$  in which one or several, or a plurality, of amino acid residues have been deleted, substituted, or inserted or added.

[0016]

As methods of introducing deletion, substitution, or insertion, there can be used site-directed mutagenesis that alters the corresponding gene (Hashimoto-Gotoh, Gene (1985) 152, 271-275, Zoller, Methods Enzymol. (1983) 100, 468-500, Kramer, Nucleic Acids Res. (1984) 12, 9441-8456, Kunkel, Proc. Natl. Acad. Sci. USA (1985) 82, 489-492, "New Cell Engineering

Experimental Protocol" edited by Dept. of Oncology, Inst. of Medical Science, Univ. of Tokyo (1993) pp. 241-248).

[0017]

It is also possible to use "Site-Directed Mutagenesis System" (GIBCO-BRL) and "QuickChange Site-Directed Mutagenesis Kit" (Stratagene) employing commercially available PCR. Amino acid mutations in proteins may sometimes take place in nature. That such a protein, in which mutation has been introduced, has an activity equal to the original protein has been shown in Mark, Proc. Natl. Acad. Sci. USA (1984) 81, 5662-5666.

[0018]

In the substitution of amino acid residues, it is preferred to substitute between amino acids whose properties are conserved. For example, substitution is preferred between hydrophobic amino acids (A, I, L, M, F, P, W, Y, V), hydrophilic amino acids (R, D, N, C, E, Q, G, H, K, S, T), amino acids having aliphatic side chains (G, A, V, L, I, P), amino acids having hydroxyl group-containing side chains (S, T, Y), amino acids having sulfur-containing side chains (C, M), amino acids having carboxylic acid- and amide-containing side chains (D, N, E, Q), amino acids having base-containing side chains (R, K, H), and amino acids having aromatic group-containing side chains (H, F, Y, W).

[0019]

Furthermore, as mutants, peptide fragments of interferon- $\alpha$  or interferon- $\gamma$  may be used. In particular, peptide fragments that have binding sites with interferon- $\alpha$  or interferon- $\gamma$  receptors. Preferably they are peptides comprised of 100 or more, more preferably 130 or more, still more preferably 150, and most preferably 160 or more contiguous amino acid residues.

[0020]

#### IRF-2 protein

Interferon regulatory factor (IRF)-1 and -2 were identified as a transcription regulatory factor of the IFN- $\beta$

gene. IRF-1 and -2 are known to bind to the same gene regulatory sequence: IRF-1 and IRF-2 act in an antagonistic manner in that IRF-1 acts as a transcription activation factor, whereas IRF-2 acts as a transcription suppressing factor. The NIH3T3 cells in which IRF-2 is highly expressed has been demonstrated to exhibit enhanced cell saturation density, colony formation in the methylcellulose gel, and a tumorigenic property in nude mice, and IRF-2 acts as an oncogene.

[0021]

On the other hand, recent advances in research have indicated that IRF-2 is required for the expression of histone H4 that acts for the control of cell cycle. IRF-2 is also shown to increase the expression of vascular cell adhesion molecule-1 (VCAM-1) in muscle cells, and it is becoming increasingly clear that the acid region (182 to 218) is involved in the activation of VCAM-1. Based on this, it is known that IRF-2 not only acts as a transcription regulatory factor but as a transcription activation factor.

[0022]

#### Hybridoma

The hybridoma produced by the antibody for use in the present invention can be basically constructed using a known technology as described below. Thus, HM1.24 antigen protein or a HM1.24 antigen-expressing cell IL-6 may be used as a sensitizing antigen and is immunized in the conventional method of immunization. The immune cells thus obtained are fused with known parent cells in the conventional cell fusion process, and then monoclonal antibody-producing cells are screened by the conventional screening method to construct the desired hybridoma.

[0023]

Specifically, monoclonal antibody may be obtained in the following manner. For example, as the HM1.24 antigen-expressing cell which is the sensitizing antigen to obtain the antibody, a human multiple myeloma cell line KPMM2 (Japanese

Unexamined Patent Publication (Kokai) No. 7-236475) and KPC-32 (Goto, T. et al., Jpn. J. Clin. Hematol. (1991) 32, 1400) can be used. As the sensitizing antigen, it is also possible to use a protein having the amino acid sequence as set forth in SEQ ID NO: 1 or a peptide or a polypeptide containing an epitope recognized by anti-HM1.24 antibody.

[0024]

The cDNA of the protein having the amino acid sequence as set forth in SEQ ID NO 1 used as the sensitizing antigen is inserted into the XbaI cleavage site of the pUC19 vector to prepare a plasmid pRS38-pUC19. *E. coli* having the plasmid pRS38-pUC19 has been internationally deposited under the provisions of the Budapest Treaty as *Escherichia coli* DH5 $\alpha$  (pRS38-pUC19) on October 5, 1993 with the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, of 1-3, Higashi 1-chome, Tsukuba city, Ibaraki Pref., Japan, as FERM BP-4434 (Japanese Unexamined Patent Publication (Kokai) No. 7-196694). Using the cDNA fragment contained in this plasmid pRS38-pUC19, a peptide or a polypeptide that contains an epitope recognized by anti-HM1.24 antibody can be constructed by a gene engineering technology.

[0025]

Mammals to be immunized with the sensitizing antigen are not specifically limited, and they are preferably selected in consideration of their compatibility with the parent cell for use in cell fusion. They generally include rodents such as mice, rats, and hamsters.

Immunization of animals with a sensitizing antigen is carried out using a known method. A general method, for example, involves the intraperitoneal or subcutaneous administration of a sensitizing antigen to the mammal.

[0026]

Specifically, a sensitizing antigen which has been diluted and suspended in an appropriate amount of phosphate buffered saline (PBS) or physiological saline etc. is mixed, as desired, with an appropriate amount of a common adjuvant,

for example Freund's complete adjuvant. After being emulsified, it is preferably administered to the mammal for several times every 4 to 21 days. Alternatively, a suitable carrier may be used at the time of immunization of the sensitizing antigen.

After immunizing in this manner and confirming the increase in the desired antibody levels in the serum, immune cells are harvested from the mammal and are subjected to cell fusion.

[0027]

The mammalian myeloma cells as the other parent cells which are subjected to cell fusion with the above-mentioned immune cells preferably include various known cell lines such as P3X63Ag8.653 (J. Immunol. (1979) 123: 1548-1550), P3X63Ag8U.1 (Current Topics in Microbiology and Immunology (1978) 81: 1-7), NS-1 (Kohler, G. and Milstein, C., Eur. J. Immunol. (1976) 6: 511-519), MPC-11 (Margulies, D.H. et al., Cell (1976) 8: 405-415), SP2/0 (Shulman, M. et al., Nature (1978) 276: 269-270), FO (de St. Groth, S. F. et al. J. Immunol. Methods (1980) 35: 1-21), S194 (Trowbridge, I. S., J. Exp. Med. (1978) 148: 313-323), R210 (Galfre, G. et al., Nature (1979) 277: 131-133) and the like.

[0028]

Cell fusion between the above immune cells and the myeloma cells may be essentially conducted in accordance with a known method such as is described in Milstein et al. (Kohler, G. and Milstein, C., Methods Enzymol. (1981) 73: 3-46) and the like.

More specifically, the above cell fusion is carried out in the conventional nutrient broth in the presence of, for example, a cell fusion accelerator. As the cell fusion accelerator, for example, polyethylene glycol (PEG), Sendai virus (HVJ) and the like may be used, and, in addition, an adjuvant such as dimethyl sulfoxide etc. may be added as desired to enhance the efficiency of the fusion.

[0029]

The preferred ratio of the immune cells and the myeloma cells to be used is, for example, 1 to 10 times more immune cells than the myeloma cells. Examples of culture media to be used for the above cell fusion include RPMI1640 medium and MEM culture medium suitable for the growth of the above myeloma cell lines, and the conventional culture medium used for this type of cell culture. A serum supplement such as fetal calf serum (FCS) may be added.

[0030]

In cell fusion, predetermined amounts of the above immune cells and the myeloma cells are mixed well in the above culture liquid, to which a PEG solution previously heated to about 37°C, for example a PEG solution with a mean molecular weight of about 1000 to 6000, is added at a concentration of 30 to 60% (w/v), and mixed to obtain the desired fusion cells (hybridomas). Then by repeating the sequential addition of a suitable culture liquid and centrifugation to remove the supernatant, cell fusion agents etc. which are undesirable for the growth of the hybridoma can be removed.

[0031]

Said hybridoma is selected by culturing in a conventional selection medium, for example, the HAT culture medium (a culture liquid containing hypoxanthine, aminopterin, and thymidine). Culturing in said HAT culture medium is continued generally for a period of time sufficient to effect killing of the cells (non-fusion cells) other than the desired hybridoma, generally several days to several weeks. Then, the conventional limiting dilution method is conducted in which the hybridomas that produce the desired antibody are screened and cloned.

[0032]

In addition to obtaining the above hybridoma by immunizing an animal other than the human with an antigen, it is also possible to sensitize human lymphocytes in vitro with HM1.24 antigen or HM1.24 antigen-expressing cells, and to allow the resulting sensitized lymphocytes to be fused with a

human-derived myeloma cell, for example U266, and thereby to obtain the desired human antibody having the activity of binding HM1.24 antigen or HM1.24 antigen-expressing cells (see Japanese Examined Patent Publication (Kokoku) No. 1-59878). Furthermore, a transgenic animal having a repertoire of human antibody genes is immunized with HM1.24 antigen or HM1.24 antigen-expressing cells to obtain the desired antibody according to the above-mentioned method (see International Patent Application WO 93/12227, WO 92/03918, WO 94/02602, WO 94/25585, WO 96/34096, and WO 96/33735).

[0033]

The monoclonal antibody-producing hybridomas thus constructed can be subcultured in the conventional culture liquid, or can be stored for a prolonged period of time in liquid nitrogen.

In order to obtain monoclonal antibodies from said hybridoma, there may be employed a method in which said hybridoma is cultured in the conventional method and the antibodies are obtained as the culture supernatant, or a method in which the hybridoma is administered to and grown in a mammal compatible with said hybridoma and the antibodies are obtained as the ascites, or other methods. The former method is suitable for obtaining high-purity antibodies, whereas the latter is suitable for a large scale production of antibodies.

[0034]

#### Monoclonal antibody

Specifically the anti-HM1.24 antibody-producing hybridoma can be constructed using the method of Goto, T. et al. (Blood (1994) 84: 1922-1930). It can be conducted by: a method in which the anti-HM1.24 antibody-producing hybridoma that was internationally deposited under the provisions of the Budapest Treaty as FERM BP-5233 on September 14, 1995 with the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, of 1-3, Higashi 1-chome, Tsukuba city, Ibaraki Pref., Japan, is intraperitoneally injected to BALB/c mice (manufactured by CLEA Japan) to obtain



the ascites, from which the anti-HM1.24 antibody is purified, or: a method in which said hybridoma is cultured in a suitable culture medium such as the RPMI1640 medium containing 10% bovine fetal serum and 5% BM-Condimed H1 (manufactured by Boehringer Mannheim), the hybridoma SFM medium (manufactured by GIBCO-BRL), the PFHM-II medium (manufactured by GIBCO-BRL) and the like, and the anti-HM1.24 antibody can be purified from the supernatant.

[0035]

#### Recombinant antibody

A recombinant antibody which was produced by the recombinant gene technology in which an antibody gene was cloned from the hybridoma and integrated into a suitable vector which was then introduced into a host can be used in the present invention as monoclonal antibody (see, for example, Carl, A. K., Borrebaeck, and James, W. Larrick, THERAPEUTIC MONOCLONAL ANTIBODIES, published in the United Kingdom by MACMILLAN PUBLISHERS LTD. 1990).

[0036]

Specifically, mRNA encoding the variable region (V) of the desired antibody is isolated from the hybridoma producing the antibody. The isolation of mRNA is conducted by preparing total RNA using, for example, a known method such as the guanidine ultracentrifuge method (Chirgwin, J.M. et al., Biochemistry (1979) 18, 5294-5299), the AGPC method (Chmczynski, P. et al., (1987) 162, 156-159), and then mRNA is purified from the total RNA using the mRNA Purification kit (manufactured by Pharmacia) and the like. Alternatively, mRNA can be directly prepared using the QuickPrep mRNA Purification Kit (manufactured by Pharmacia).

[0037]

cDNA of the V region of the antibody may be synthesized from the mRNA thus obtained using a reverse transcriptase. cDNA may be synthesized using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit and the like. Alternatively, for the synthesis and amplification of cDNA, the 5'-Ampli

FINDER RACE Kit (manufactured by Clontech) and the 5'-RACE method (Frohman, M.A. et al., Proc. Natl. Acad. Sci. U.S.A. (1988) 85, 8998-9002; Belyavsky, A. et al., Nucleic Acids Res. (1989) 17, 2919-2932) that employs PCR may be used. The desired DNA fragment is purified from the PCR product obtained and may be ligated to vector DNA. Moreover, a recombinant vector is constructed therefrom and then is introduced into *E. coli* etc., from which colonies are selected to prepare the desired recombinant vector. The base sequence of the desired DNA may be confirmed by a known method such as the dideoxy method.

[0038]

Once the DNA encoding the V region of the desired antibody has been obtained, it may be ligated to DNA encoding the constant region (C region) of the desired antibody, which is then integrated into an expression vector. Alternatively, the DNA encoding the V region of the antibody may be integrated into an expression vector which already contains DNA encoding the C region of the antibody.

In order to produce the antibody for use in the present invention, the antibody gene is integrated as described below into an expression vector so as to be expressed under the control of the expression regulatory region, for example an enhancer and/or a promoter. Subsequently, the expression vector may be transformed into a host cell and the antibody can then be expressed therein.

[0039]

#### Altered antibody

In accordance with the present invention, artificially altered recombinant antibody such as chimeric antibody and humanized antibody can be used for the purpose of lowering heterologous antigenicity against humans. These altered antibody can be produced using known methods.

Chimeric antibody can be obtained by ligating the thus obtained DNA encoding the V region of antibody to DNA encoding the C region of human antibody, which is then integrated into

an expression vector and introduced into a host for production of the antibody therein (see European Patent Application EP 125023, and International Patent Application WO 96/02576). Using this known method, chimeric antibody useful for the present invention can be obtained.

[0040]

For example, *E. coli* having the plasmid that contains the L chain and the H chain of chimeric anti-HM1.24 antibody has been internationally deposited under the provisions of the Budapest Treaty as *Escherichia coli* DH5 $\alpha$  (pUC19-1.24L-gk) and *Escherichia coli* DH5 $\alpha$  (pUC19-1.24H-gyl), respectively, on August 29, 1996 with the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, of 1-3, Higashi 1-chome, Tsukuba city, Ibaraki Pref., Japan, as FERM BP-5646 and FERM BP-5644, respectively (see Japanese Patent Application No. 8-264756).

[0041]

Humanized antibody which is also called reshaped human antibody has been made by transplanting the complementarity determining region (CDR) of antibody of a mammal other than the human, for example mouse antibody, into the CDR of human antibody. The general recombinant DNA technology for preparation of such antibodies is also known (see European Patent Application EP 125023 and International Patent Application WO 96/02576).

[0042]

Specifically, a DNA sequence which was designed to ligate the CDR of mouse antibody with the framework region (FR) of human antibody is synthesized by the PCR method from several divided oligonucleotides having sections overlapping with one another at the ends thereof. The DNA thus obtained is ligated to the DNA encoding the C region of human antibody and then is integrated into an expression vector, which is introduced into a host for antibody production (see European Patent Application EP 239400 and International Patent Application WO 96/02576).

[0043]

For the FR of human antibody ligated through CDR, the complementarity determining region that forms a favorable antigen binding site is selected. When desired, amino acids in the framework region of the antibody variable region may be substituted so that the complementarity determining region of reshaped human antibody may form an appropriate antigen binding site (Sato, K. et al., Cancer Res. (1993) 53, 851-856).

For example, *E. coli* having the plasmid that contains the L chain and the H chain of humanized anti-HM1.24 antibody has been internationally deposited under the provisions of the Budapest Treaty as *Escherichia coli* DH5 $\alpha$  (pUC19-RVLa-AHM-gk) and *Escherichia coli* DH5 $\alpha$  (pUC19-RVHr-AHM-gyl), respectively, on August 29, 1996 with the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, of 1-3, Higashi 1-chome, Tsukuba city, Ibaraki Pref., Japan, as FERM BP-5645 and FERM BP-5643, respectively (See Japanese Patent Application No. 8-264756).

[0044]

For chimeric antibody or humanized antibody, the C region of human antibody is used, and as the C region of human antibody that exhibits cytotoxic activity, human C $\gamma$ , for example C $\gamma$ 1, C $\gamma$ 2, C $\gamma$ 3, and C $\gamma$ 4 can be used. Among them, antibody having C $\gamma$ 1 and C $\gamma$ 3 in particular has potent cytotoxic activity, i.e. ADCC activity and CDC activity, and is used preferably in the present invention.

[0045]

Chimeric antibody consists of the variable region of antibody derived from a mammal other than the human and the C region derived from human antibody, whereas humanized antibody consists of the complementarity determining region of antibody derived from a mammal other than the human and the framework region (FR) and the C region of antibody derived from human antibody. Accordingly, antigenicity thereof in the human body has been reduced so that they are useful as the active

ingredient of the therapeutic agents of the present invention.

A preferred embodiment of the humanized antibody for use in the present invention includes humanized anti-HM1.24 antibody (see Japanese Patent Application No. 8-264756).

[0046]

#### Expression and production

Antibody genes constructed as described above may be expressed and obtained in a known method. In the case of mammalian cells, expression may be accomplished using an expression vector containing a commonly used useful promoter, the antibody gene to be expressed, and DNA in which the poly A signal has been operably linked at 3' downstream thereof or a vector containing said DNA. Examples of the promoter/enhancer include human cytomegalovirus immediate early promoter/enhancer.

[0047]

Additionally, as a promoter/enhancer which can be used for the expression of antibody for use in the present invention, there can be used viral promoters/enhancers such as retrovirus, polyoma virus, adenovirus, and simian virus 40 (SV40), and promoters/enhancers derived from mammalian cells such as human elongation factor 1 $\alpha$  (HEF1 $\alpha$ ).

For example, expression may be readily accomplished by the method of Mulligan et al. (Nature (1979) 277, 108) when SV40 promoter/enhancer is used, or by the method of Mizushima et al. (Nucleic Acids Res. (1990) 18, 5322) when HEF1 $\alpha$  promoter/enhancer is used.

[0048]

In the case of *E. coli*, expression may be conducted by operably linking a commonly used useful promoter, a signal sequence for antibody secretion, and the antibody gene to be expressed, followed by expression thereof. As the promoter, for example, there can be mentioned lacZ promoter and araB promoter. The method of Ward et al. (Nature (1998) 341, 544-546; FASEB J. (1992) 6, 2422-2427) may be used when lacZ promoter is used, and the method of Better et al. (Science

(1988) 240, 1041-1043) may be used when araB promoter is used.  
[0049]

As a signal sequence for antibody secretion, when produced in the periplasm of *E. coli*, the pelB signal sequence (Lei, S.P. et al., J. Bacteriol. (1987) 169, 4379) can be used. After separating the antibody produced in the periplasm, the structure of the antibody is appropriately refolded before use (see, for example, WO 96/30394).  
[0050]

As an origin of replication, there can be used those derived from SV40, polyoma virus, adenovirus, bovine papilloma virus (BPV) and the like. Furthermore, for the amplification of the gene copy number in the host cell system, expression vectors can include as selectable markers the aminoglycoside transferase (APH) gene, the thymidine kinase (TK) gene, *E. coli* xanthine guaninephosphoribosyl transferase (Ecogpt) gene, the dihydrofolate reductase (dhfr) gene and the like.

For the production of antibody for use in the present invention, any production system can be used. The production system of antibody preparation comprises the in vitro and the in vivo production system. As the in vitro production system, there can be mentioned a production system which employs eukaryotic cells and the production system which employs prokaryotic cells.

[0051]

When the eukaryotic cells are used, there are the production systems which employ animal cells, plant cells, and fungal cells. Known animal cells include (1) mammalian cells such as CHO cells, COS cells, myeloma cells, baby hamster kidney (BHK) cells, HeLa cells, and Vero cells, (2) amphibian cells such as *Xenopus* oocytes, or (3) insect cells such as sf9, sf21, and Tn5. Known plant cells include, for example, those derived from the genus *Nicotiana*, more specifically cells derived from *Nicotiana tabacum*, which is subjected to callus culture. Known fungal cells include yeasts such as the genus *Saccharomyces*, more specifically *Saccharomyces*

cereviceae, or filamentous fungi such as the genus *Aspergillus*, more specifically *Aspergillus niger*.

[0052]

When the prokaryotic cells are used, there are the production systems which employ bacterial cells. Known bacterial cells include *Escherichia coli* (*E. coli*), and *Bacillus subtilis*.

By introducing, via transformation, the gene of the desired antibody into these cells and culturing the transformed cells in vitro, the antibody can be obtained. Culturing is conducted in the known methods. For example, as the culture liquid, DMEM, MEM, RPMI1640, and IMDM can be used, and serum supplements such as fetal calf serum (FCS) may be used in combination. In addition, antibodies may be produced in vivo by implanting cells into which the antibody gene has been introduced into the abdominal cavity of an animal and the like.

[0053]

As further in vivo production systems, there can be mentioned those which employ animals and those which employ plants. When animals are used, there are the production systems which employ mammals and insects.

As mammals, goats, pigs, sheep, mice, and cattle can be used (Vicki Glaser, SPECTRUM Biotechnology Applications, 1993). Also as insects, silkworms can be used.

When plants are used, tobacco, for example, can be used.

[0054]

Antibody genes are introduced into these animals or plants, and the antibodies are produced in such animals or plants, and recovered therefrom. For example, an antibody gene is inserted into the middle of the gene encoding protein which is inherently produced in the milk such as goat  $\beta$  casein to prepare fusion genes. DNA fragments containing the fusion gene into which the antibody gene has been inserted are injected into a goat embryo, and the embryo is introduced into a female goat. The desired antibody is obtained from the milk

produced by the transgenic goat borne to the goat who received the embryo or offsprings thereof. In order to increase the amount of milk containing the desired antibody produced by the transgenic goat, hormones may be given to the transgenic goat as appropriate. (Ebert, K.M. et al., Bio/Technology (1994) 12, 699-702).

[0055]

When silkworms are used, baculovirus into which the desired antibody gene has been inserted is infected to the silkworm, and the desired antibody can be obtained from the body fluid of the silkworm (Susumu, M. et al., Nature (1985) 315, 592-594). Moreover, when tobacco is used, the desired antibody gene is inserted into an expression vector for plants, for example pMON 530, and then the vector is introduced into a bacterium such as *Agrobacterium tumefaciens*. The bacterium is then infected to tobacco such as *Nicotiana tabacum* to obtain the desired antibody from the leaves of the tobacco (Julian, K.-C. Ma et al., Eur. J. Immunol. (1994) 24, 131-138).

[0056]

When antibody is produced in the in vitro or in vivo production systems, as described above, DNA encoding the heavy chain (H chain) or the light chain (L chain) of antibody may be separately integrated into an expression vector and the hosts are transformed simultaneously, or DNA encoding the H chain and the L chain may be integrated into a single expression vector and the host is transformed therewith (see International Patent Application WO 94-11523).

[0057]

The antibody produced as described above can be bound to various molecules such as polyethylene glycol (PEG) for use as a modified antibody. "Antibody" as used herein includes these modified antibodies. In order to obtain these modified antibody, the antibody obtained may be chemically modified. These methods have already been established in the field of the art.



[0058]

Separation and purification of antibody

Antibodies produced and expressed as described above can be separated from the inside or outside of the cell or from the host and then may be purified to homogeneity. Separation and purification of the antibody for use in the present invention may be accomplished by affinity chromatography. As the column used for such affinity chromatography, there can be mentioned Protein A column and Protein G column. Examples of the column employing Protein A column are Hyper D, POROS, Sepharose F.F. and the like.

[0059]

Alternatively, methods for separation and purification conventionally used for proteins can be used without any limitation. Separation and purification of the antibody for use in the present invention may be accomplished by combining, as appropriate, chromatography other than the above-mentioned affinity chromatography, filtration, ultrafiltration, salting-out, dialysis and the like. Chromatography includes, for example, ion exchange chromatography, hydrophobic chromatography, gel-filtration and the like.

[0060]

Determination of antibody concentration

The concentration of antibody obtained in the above method can be determined by the measurement of absorbance or by ELISA and the like. Thus, when absorbance measurement is employed, the antibody for use in the present invention or a sample containing the antibody is appropriately diluted with PBS(-) and then the absorbance is measured at 280 nm, followed by calculation using the absorption coefficient of 1.35 OD at 1 mg/ml. When the ELISA method is used, measurement is conducted as follows. Thus, 100  $\mu$ l of goat anti-human IgG (manufactured by BIO SOURCE) diluted to 1  $\mu$ g/ml in 0.1 M bicarbonate buffer, pH 9.6, is added to a 96-well plate (manufactured by Nunc), and is incubated overnight at 4°C to immobilize the antibody.

[0061]

After blocking, 100  $\mu$ l each of appropriately diluted antibody of the present invention or a sample containing the antibody, or 100  $\mu$ l of human IgG (manufactured by CAPPEL) as the standard is added, and incubated at room temperature for 1 hour. After washing, 100  $\mu$ l of 5000-fold diluted alkaline phosphatase-labeled anti-human IgG antibody (manufactured by BIO SOURCE) is added, and incubated at room temperature for 1 hour. After washing, the substrate solution is added and incubated, followed by the measurement of absorbance at 405 nm using the MICROPLATE READER Model 3550 (manufactured by Bio-Rad) to calculate the concentration of the desired antibody.

[0062]

#### FCM analysis

Reactivity of the antibody of the present invention with lymphocytes may be examined by flow cytometry (FCM) analysis. As the cells, established cell lines or freshly isolated cells can be used. As established cell lines, there may be used myeloma-derived RPMI8226 (ATCC CCL 155), myeloma-derived U266 (ATCC TIB 196), myeloma-derived KPMM2, myeloma-derived KPC-32, and plasmacytoma-derived ARH-77 (ATCC CRL 1621), and the like.

[0063]

After washing the above cells in PBS(-), 100  $\mu$ l of antibody or a control antibody diluted to 25  $\mu$ g/ml in the FACS buffer (PBS(-) containing 2% bovine fetal serum and 0.05% sodium azide) is added thereto, which is then incubated on ice for 30 minutes. After washing with the FACS buffer, 100  $\mu$ l of 25  $\mu$ g/ml FITC-labeled goat anti-mouse antibody (GAM, manufactured by Becton Dickinson) is added thereto, which is then incubated on ice for 30 minutes. After washing with the FACS buffer, the cells are suspended in 600  $\mu$ l or 1 ml of the FACS buffer, and each cell may be measured for its fluorescence intensity using the FACScan (manufactured by Becton Dickinson).

[0064]

Screening method

In order to screen the expression enhancer of HM1.24 antigen, for example, the cells that have not been stimulated and that are not expressing HM1.24 antigen or the cells that at least are expressing the antigen are determined using FCM analysis. For example, the cells described in Examples and a test substance are incubated for 1-2 days, and then stained with mouse anti-human HM1.24 antibody as a primary antibody. The cells are washed and further stained with FITC-labeled anti-mouse IgG antibody as a secondary antibody. Finally, after washing the cells, the fluorescence intensity of FITC is measured by flow cytometry.

[0065]

Furthermore, instead of the above indirect staining, FCM analysis by direct staining may be used in which the cells are treated with a high concentration of immunoglobulin, and then stained, after blocking Fc receptors, with FITC-labeled anti-human HM1.24 antibody.

It is also possible to screen the expression enhancers of HM1.24 antigen using a reporter gene assay and using the HM1.24 promoter sequence. As the reporter gene, luciferase can be used. A plasmid is constructed that contains the HM1.24 promoter sequence upstream of the reporter gene, after which it is transformed into the cells, and the cells obtained are cultured with a test substance for 1-2 days, and the cells recovered are subjected to luciferase assay to screen drugs that enhance the expression of HM1.24 antigen.

[0066]

Cytotoxic activity

Measurement of ADCC activity

The antibody for use in the present invention is one which has, for example, an ADCC activity as the cytotoxic activity.

According to the present invention, the ADCC activity on myeloma cells can be measured in the following manner. First,

mononuclear cells are isolated as the effector cells from human peripheral blood or bone marrow by the gravity centrifuge method.

[0067]

As the target cells (Target cell: T), RPMI8226 (ATCC CCL 155), U266 (ATCC TIB 196), KPMM2, KPC-32, ARH-77 (ATCC CRL 1621) or the like are labeled with  $^{51}\text{Cr}$  to be prepared as the target cells. Subsequently, to the labeled target cells is added the antibody to be measured for the ADCC activity and incubated. Effector cells at a suitable ratio to the target cells are then added and incubated.

[0068]

After the incubation, the supernatant is removed and measured for radioactivity using a gamma counter, whereupon 1% NP-40 can be used for measurement of the maximum free radioactivity. The cytotoxic activity (%) can be calculated as  $(A-C)/(B-C) \times 100$ , in which A is radioactivity (cpm) liberated in the presence of the antibody, B is radioactivity (cpm) liberated by NP-40, and C is radioactivity (cpm) liberated by the medium alone containing no antibody.

[0069]

#### Enhancement of cytotoxic activity

In order to exhibit cytotoxic activity such as an ADCC activity, it is preferred to use  $\text{C}_\gamma$ , in particular  $\text{C}_{\gamma 1}$  and  $\text{C}_{\gamma 3}$  as the constant region (C region) of antibody in humans. Furthermore, a more potent ADCC activity or CDC activity can be induced by adding, altering, or modifying part of the amino acids in the C region of antibody.

[0070]

By way of example, there can be mentioned the construction of an IgM-like polymer of IgG by amino acid substitution (Smith, R.I.F. & Morrison, S.L. BIO/TECHNOLOGY (1994) 12, 683-688), the construction of an IgM-like polymer of IgG by amino acid addition (Smith, R.I.F. et al., J. Immunology (1995) 154, 2226-2236), the expression of a tandemly-ligated gene encoding L chain (Shuford, W. et al.,

Science (1991) 252, 724-727), the dimerization of IgG by amino acid substitution (Caron, P.C. et al., J. Exp. Med. (1992) 176, 1191-1195, Shopes, B., J. Immunology (1992) 148, 2918-2922), the dimerization of IgG by chemical modification (Wolff, E. A. et al., Cancer Res. (1993) 53, 2560-2565), and the introduction of the effector function by altering an amino acid(s) in the hinge region of antibody (Norderhaug, L. et al., Eur. J. Immunol. (1991) 21, 2379-2384) and the like.  
[0071]

These can be accomplished by means of the oligomer site-specific mutagenesis using a primer, the addition of a base sequence using a restriction enzyme cleavage site, and the use of a chemical modifier that creates a covalent bond.

[0072]

[EXAMPLES]

Example 1. Enhancement of the amount expressed of HM1.24 antigen in myeloma cells by interferon- $\alpha$

A human myeloma cell line U266 (ATCC TIB 196) and myeloma cells derived from the bone marrow of a patient with multiple myeloma were cultured in a RPMI1640 medium (Sigma, St Louis, MO, USA) containing 10% fetal bovine serum (Whittaker Bioproducts, Inc., Walkersville, MD, USA) in a 5% carbon dioxide incubator at 37°C. The hybridoma that produces mouse anti-HM1.24 antibody has been internationally deposited as FERM BP-5233 (deposition date: April 27, 1995) with the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, of 1-3, Higashi 1-chome, Tsukuba city, Ibaraki Pref.

[0073]

Myeloma cells ( $1 \times 10^5$ /ml) were cultured in the presence or absence of 1000 U/ml of the natural type interferon- $\alpha$  (Otsuka Pharmaceutical, Tokyo) for 48 hours, and changes in HM1.24 antigen (the base sequence encoding this is shown in SEQ ID NO: 1) were determined by flow cytometry. After the cells were washed with phosphate buffer (Gibco BRL, Grand Island, NY, USA) supplemented with 0.1% bovine serum albumin

(Sigma, St Louis, MO, USA) and 0.02% sodium azide, they were suspended into PBS (100  $\mu$ l) supplemented with human immunoglobulin (3 mg/ml, Green Cross, Osaka), and were allowed to react at 4°C for 15 minutes.

[0074]

Thereafter, 2  $\mu$ l of FITC-human IgG1 (1 mg/ml) or FITC-anti-HM1.24 antibody (1 mg/ml) was added to stain at 4°C for 60 minutes. When the patient's myeloma cells were used, 20  $\mu$ l of PE-anti-CD38 (Becton Dickinson, San Jose, CA, USA) was added for double staining to identify the myeloma cell. After staining, the cells were washed twice with PBS, and were stored in PBS containing 1% paraformaldehyde (Wako Pure Chemical Industries, Ltd., Osaka). Subsequently, the expression of HM1.24 antigen was analyzed using a flow cytometer (EPICS XL, Coulter, Hialeah, FL, USA).

[0075]

As a result, a myeloma cell line U266 (Figure 1) and the patient's myeloma cells (Figure 2) were expressing HM1.24 antigen at a condition of no stimulation, and the stimulation with interferon- $\alpha$  further increased the amount expressed of HM1.24 antigen.

Interferon- $\alpha$  further enhanced the expression of HM1.24 antigen in the myeloma cell, and increased the number of anti-HM1.24 antibodies that bind to the myeloma cell. Since the therapeutic anti-tumor effect by anti-HM1.24 antibody is proportional to the number of binding antibodies, treatment with anti-HM1.24 antibody after the amplified of interferon- $\alpha$  is expected to become a therapy that enhances the therapeutic effect by antibody and further enhances effectiveness.

[0076]

Example 2. Analysis of the expression function of HM1.24 antigen by the reporter gene analysis

In order to investigate whether the expression induction of antigen is regulated by the HM1.24 promoter region, the reporter gene at the promoter region was analyzed.

The gene (SEQ ID NO: 3) of the HM1.24 promoter region was obtained by PCR cloning. Genomic DNA was prepared from human peripheral blood mononuclear cells using the DNazol reagent (GIBCO). With the genomic DNA obtained as the template, using primer HM2k (aaaggtaccagctgtctttctgtctgtcc) (SEQ ID NO: 4) and BST2B (atagtcatacgaagtagatgccatccag) (SEQ ID NO: 5), PCR (94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 30 cycles) was performed using TaKaRa Taq (Takara Shuzo, Ohtsu) in a Thermal Cycler 480 (Perkin-Elmer, CA, USA).

[0077]

An about 2 kb fragment obtained was treated with restriction enzymes KpnI and BglII (Takara Shuzo), and was cloned into the KpnI-BglII site of a reporter gene plasmid pGL3-basic (Promega, WI, USA) using the DNA ligation kit ver. II (Takara Shuzo) to transform into competent *E. coli* JM109 (Nippongene). The transformed *E. coli* was cultured at 37°C in the LB medium containing 100 µg/ml ampicillin, and the plasmid was prepared using the QIAGEN plasmid maxi kit (QIAGEN, Hilden, Germany).

[0078]

The plasmid HM-2k/GL3 obtained was treated with restriction enzymes KpnI and XhoI, from which a deletion clone was constructed using the deletion kit (Takara Shuzo) for a kilo-sequence to obtain a plasmid HM-493/GL3 containing from the transcription initiation point to 493 bp upstream. Furthermore, HM-2k/GL3 was treated with restriction enzymes KpnI and AflIII, from which a deletion clone was constructed as described above, and HM-151/GL3 and HM-77/GL3 containing from the transcription initiation point to -151 bp or -77 bp upstream.

[0079]

For the introduction of the plasmid into the cell, the polyethyleneimine-Transferrinfection Kit (Tf PEI-Kit) (Bender MedSystems, Vienna, Austria) was used, and for the luciferase assay the Dual-Luciferase Reporter Assay System (Promega) was used. The cell line was cultured overnight in RPMI-1640

medium containing 50  $\mu$ M Defferrioxamine and 10% FBS. In order to form a complex of the plasmid to be introduced with Tf-PEI, a mixture of the reporter gene plasmid at a final concentration of 20  $\mu$ g/ml, 0.4  $\mu$ g/ml of pRL-SV40, and 1  $\mu$ g/ml of Tf-PEI reagent was prepared and was incubated at room temperature for 20 minutes.  $5 \times 10^5$  cells/ml of cells were added at three volumes of the Tf-PEI/plasmid mixture, and was incubated at 37°C for four hours, washed with the medium, and 100  $\mu$ l per well at a concentration of  $2 \times 10^5$  cells/ml was cultured in a 96-well flat-bottomed plate.

[0080]

IFN- $\alpha$  was added to a final concentration of 0, 10, 100, or 1000 U/ml, which was cultured at 37°C for two days. After the cells were washed in PBS(-), they were dissolved in 20  $\mu$ l of the Passive Lysis Buffer, six  $\mu$ l of which was applied to the C96 White Polysorp Fluoronunc plate (Nunc). Using the Luminoskan (Labsystems), luminescence intensity was measured for Firefly and Renila at 30  $\mu$ l of the substrate solution and a measurement time of 10 seconds. The measured values were corrected by Firefly/Renila, and the relative activity was determined with the control (medium) as one.

[0081]

As a result, the luciferase activity of the reporter was increased in a IFN- $\alpha$  concentration dependent manner for both of the upstream 2 kbp and 493 bp, confirming that the enhanced transcription activity of the promoter region causes the expression induction of the antigen (Figure 3).

Furthermore, the result of an experiment in which the upstream 151 bp and 77 bp reporter plasmid of the transcription initiation point were used, an enhanced luciferase activity by IFN- $\alpha$  stimulation was observed for the upstream 151 bp reporter plasmid. On the other hand, no changes in activity were noted by IFN- $\alpha$  stimulation in the upstream 77 bp reporter plasmid (Figure 4). In the region of



77-151 bp, a sequence having a high homology with GAS element and ISRE was present and, as it is a transcription regulatory factor that is activated in response to IFN- $\alpha$  stimulation, the transcription regulatory factor of the IRF family was shown to be involved in the activity.

[0082]

Example 3. Enhancement of the amount expressed of HM1.24 antigen in myeloma cells by interferon- $\gamma$

According to the method described in Example 1, 1000 U/ml of the natural type interferon- $\gamma$  (R & D System) was used for analysis. As a result, increases in the amount expressed of HM1.24 antigen were observed in both of the myeloma cell line U266 (Figure 5) and the patient's myeloma cells (Figure 6) as for interferon- $\alpha$ .

[0083]

Example 4. Binding of IRF-2 to the HM1.24 promoter region

In order to identify the transcription factor that binds to the HM1.24 promoter region, the Electrophoresis Mobility Shift Assay (EMSA) with the HM1.24 promoter region as the probe was performed as follows to identify IRF-2 as the binding factor.

[0084]

(1) Preparation of nuclear extract

The myeloma cells U266-B1 (ATCC-TIB196) were cultured in the RPMI-1640 medium (GIBCO-BRL) containing 10% FBS (HyClone) at 37°C in a 5% CO<sub>2</sub> incubator. In order to stimulate the cells by interferon- $\alpha$  (IFN- $\alpha$ ) (Pepro Tech EC), IFN- $\alpha$  was added to the medium to a final concentration of 1000 U/ml, and the cells were recovered at 30 minutes, two hours, four hours, and eight hours after the addition. The cells were suspended into cold PBS (-), centrifuged at 1,000 rpm to discard the supernatant, and suspended in a 10 mM Tris, 10 mM NaCl, and 6 mM MgCl<sub>2</sub> solution.

[0085]

After allowing to stand in ice for five minutes, centrifugation was repeated, and the supernatant was discarded. The cells were suspended into 10 mM Tris, 10 mM NaCl, 6 mM MgCl<sub>2</sub>, 1 mM DTT, 0.4 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>. The cells were homogenized on ice using a glass homogenizer, centrifuged at 6000 g for three minutes, and the supernatant was discarded. The cells were suspended into the extraction buffer (20% glycerol, 20 mM HEPES, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.2 mM PMSF, 1 mM DTT, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mg/ml aprotinin, and 5 mg/ml leupeptin), and then was allowed to stand in ice for 20 minutes. It was centrifuged at 12000 g for 10 minutes, and the supernatant was recovered.

[0086]

#### (2) Preparation of the labeled probe

As the probe, ISRE2 was constructed that contains a sequence (ttcccgagaaa (SEQ ID NO: 10)) having a homology with GAS (IFN- $\gamma$  activation site: the GAS consensus sequence is ttncnnnaa (SEQ ID NO: 8)) and ISRE (IFN- $\alpha$  stimulation response factor: the ISRE consensus sequence is ngaaanngaact (SEQ ID NO: 9)), and ggaaactgaaact (SEQ ID NO: 11) at the HM1.24 promoter region. Thus, oligo DNA ISRE-F2 (aatttctgaggaaactgaaactgaaaacct (SEQ ID NO: 12)) and ISRE-R2 (aattagggttttcagtttcagtttcccaga (SEQ ID NO: 13)) were mixed and annealed to form a double stranded DNA probe ISRE2.

[0087]

Furthermore, oligo DNA adp-1 (catggcatctacttcgtatgactattgcagagtgcc (SEQ ID NO: 14)) and adp-2 (catgggcactctgcaatagtcatacgaagtagatgc (SEQ ID NO: 15)) were mixed and annealed to form an unrelated probe adp. Probes were labeled using the Band Shift Kit (Amersham Pharmacia Biotech) according to the standard protocol. Thus, 50 ng of double stranded DNA constructed as above was subjected to the polymerase reaction of the Klenow fragment in a reaction solution containing [ $\alpha$ -<sup>32</sup>P]dATP (20  $\mu$ Ci) (Amersham Pharmacia Biotech) at 37°C for one hour. The solution that

completed the reaction was diluted two-fold and then was loaded to the Nick Spin Column (Amersham Pharmacia Biotech), and after centrifugation at 1600 rpm for four minutes, the solution was recovered to prepare a labeled probe.

[0088]

(3) Changes with time in the binding factor produced  
by stimulation of IFN- $\alpha$

According to the standard protocol of the Band Shift Kit (Amersham Pharmacia Biotech, NJ, USA), the following procedure was performed. To 5  $\mu$ g of the extracts temporally prepared in the above (1) were added 2  $\mu$ l of the 10 X binding buffer (100 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 mM DTT), 4  $\mu$ l of 50% glycerol, 1  $\mu$ l of 1% NP-40, and 1  $\mu$ l of poly(dI-dC)/poly(dI-dC), and 2  $\mu$ l of the  $^{32}$ P labeled ISRE-2 probe prepared in the above (2) was added, to which water was added to a total volume of 20  $\mu$ l, and this reaction mixture was incubated at room temperature for 20 minutes to allow for the binding of the possible binding factors that may be present in the above extract and said  $^{32}$ P labeled ISRE-2 probe.

[0089]

To 18  $\mu$ l of the reaction mixture was added 2  $\mu$ l of the 10 X stain solution (attached to the kit), which was electrophoresed in 1 X Tris-glycine buffer (25 mM Tris, 190 mM glycine, 1 mM EDTA, pH 8.1) on a 7.5% acrylamide gel, and then, after electrophoresis, the gel was attached to the filter paper to transfer protein to the filter paper. The filter paper dried with a gel drier was exposed to X-ray film to detect signals.

[0090]

For comparison, a reaction solution [(NEC-)] to which no extract was added, a reaction solution [0h] to which an extract from the cell culture that was cultured without stimulation by interferon- $\alpha$  was added, a reaction solution {8h (+cold)} in which 50 ng of a nonlabeled ISRE2 probe was

added instead of the labeled probe to the extract of 8 hour-culture, and a reaction solution [8h (+cold unrelated)] in which 50 ng of an unrelated probe adp was added to the extract of 8 hour-culture were prepared, and were processed as described above to detect signals.

The result is shown in Figure 7. As can be seen from this figure, a substance that binds to a double stranded DNA corresponding to part of the HM1.24 promoter increased with time in the U266-B1 cells cultured under the stimulation by interferon.

[0091]

(4) The identification of a transcription factor by reaction with various antibodies

In a manner similar to that described in the above (1), the myeloma cells U266-B1 (ATCC-TIB196) were cultured for eight hours in the presence of 1000 U/ml of interferon- $\alpha$  to prepare an extract. The following procedure was performed according to the standard protocol of the Band Shift Kit (Amersham Pharmacia Biotech). Thus, 2  $\mu$ g of antibody was added to 5  $\mu$ g of the extract, and incubated at room temperature for 15 minutes to obtain an extract/antibody reaction solution. To 2  $\mu$ l of the 10 X binding buffer attached to the kit, 4  $\mu$ l of 50% glycerol, 1  $\mu$ l of 1% NP-40, and 1  $\mu$ l of Poly(dI-dC)/Poly(dI-dC) were added 2  $\mu$ l of the above extract/antibody reaction solution and 2  $\mu$ l of the labeled probed prepared in the above (2), to which water was added to make the total volume 20  $\mu$ l, and the reaction mixture was incubated at room temperature for 20 minutes.

[0092]

The reaction mixture was subjected to electrophoresis as described in the above (3) to detect signals.

As the above antibody, the following antibodies (all are from Santa Cruz Biotechnology) were used:

[0093]

Anti-human STAT1 p84/p91 (E-23): (description) rabbit polyclonal antibody (SC-346X)

Anti-human STAT2 (C-20): rabbit polyclonal antibody (SC-476X)

Anti-mouse STAT3 (K-15): rabbit polyclonal antibody (SC-483X)

Anti-human ISGF-3 $\gamma$  p48 (C-20): rabbit polyclonal antibody (SC-496X)

Anti-human IRF-1 (C-20): rabbit polyclonal antibody (SC-497X)

Anti-human IRF-2 (C-19): rabbit polyclonal antibody (SC-498X)

Anti-mouse ICSAT (M-17): rabbit polyclonal antibody (SC-6059X)

[0094]

As a control, a reaction solution that uses an extract of the cells cultured without interferon stimulation [0h]; a reaction solution in which an extract of the cells cultured for eight hours under the stimulation of 1000 U/ml interferon- $\alpha$  was added, and no antibody was added [8h]; a reaction solution in which 50 ng of the nonlabeled ISRE2 probe was added the labeled ISRE2 [8h (+cold)]; a reaction solution in which 50 ng of the nonlabeled dp probe was added the labeled ISRE2 probe [8h (+cold)] and were prepared, and were processed as described above.

[0095]

The result is shown in Figure 8. As can be seen from this figure, it was shown that the component that binds to the labeled ISRE2 probe in the extract from the cells cultured under the stimulation of interferon- $\alpha$  binds only to anti-IRF-2 antibody, and the factor that binds to and thereby activates the HM1.24 promoter is a transcription factor IRF-2.

[0096]

Example 5. Confirmation of the HM1.24 promoter  
activation with IRF-2

Effect on HM1.24 promoter activity by IRF-2 co-expression was determined by the reporter gene assay using the U266 cells, and it was revealed that IRF-2 actually has the transcription activation activity of the HM1.24 promoter. In the following experiment, a myeloma cell line U266-B1 (ATCC TIB196) was used. The cells were cultured in the RPMI-1640 medium (GIBCO) (referred to hereinafter as the medium) containing 10% FBS (GIBCO BRL) in an 5% CO<sub>2</sub> incubator.

[0097]

(1) Construction of a plasmid containing the HM1.24 promoter region

The gene of the HM1.24 promoter region was obtained by PCR cloning. From human peripheral blood mononuclear cells, genomic DNA was prepared using the DNAzol reagent (GIBCO). With the genomic DNA obtained as the template, using primer HM2k (aaaggtaccagctgtcctttctgtctgtcc) (SEQ ID NO: 16) and BST2B (atagtcatacgaagtagatgccatccag) (SEQ ID NO: 17), PCR (94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 30 cycles) was performed using TaKaRa Taq (Takara Shuzo, Ohtsu) in the Thermal Cycler 480 (Perkin-Elmer, CA, USA).

[0098]

An about 2 kb fragment obtained was treated with restriction enzymes KpnI and BglII (Takara Shuzo), and was cloned into the KpnI-BglII site of a reporter gene plasmid pGL3-basic (Promega, WI, USA) using the DNA ligation kit ver. II (Takara Shuzo) to transform competent *E. coli* JM109 (Nippongene). The transformed *E. coli* was cultured at 37°C in the LB medium containing 100 µg/ml ampicillin, and a plasmid was prepared using the QIAGEN plasmid maxi kit (QIAGEN, Hilden, Germany).

[0099]

The plasmid HM-2k/GL3 obtained was treated with restriction enzymes KpnI and XhoI, from which a deletion clone was constructed using the deletion kit for kilo-sequencing (Takara Shuzo) obtain a plasmid HM-491/GL3 containing up to - 491 bp upstream of the transcription initiation point.

Furthermore, HM-2k/GL3 was treated with restriction enzymes KpnI and AflIII, from which a deletion clone was constructed as described above, and HM-151/GL3 containing -151 bp upstream of the transcription initiation point.

[0100]

Furthermore, with HM-2k/GL3 as the template, using primer 10S (tttcggtacctaattaatcctctgcctg) (SEQ ID NO: 18) and GL primer 2 (ctttatgtttttggcgtcttcca) (SEQ ID NO: 19), PCR (94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 30 cycles) was performed using TaKaRa Taq (Takara Shuzo, Ohtsu) in a Thermal Cycler 480 (Perkin-Elmer, CA, USA). The fragment obtained was treated with restriction enzymes KpnI and BglII (Takara Shuzo), and was cloned into the KpnI-BglII site of a reporter gene plasmid pGL3-basic (Promega, WI, USA) using the ligation high (Toyobo) to transform competent *E. coli* JM109 (Nippongene).

[0101]

The transformed *E. coli* was cultured at 37°C in the LB medium containing 100 µg/ml ampicillin, and a plasmid was prepared using the QIAGEN plasmid maxi kit (QIAGEN, Hilden, Germany). A plasmid HM-125/GL3 was thus obtained that contains up to 125 bp upstream of the transcription initiation point. Furthermore, with HM-2k/GL3 as the template, using primer HMP700 (aaaggtaccagaggtttacctggtatcctgg) (SEQ ID NO: 20) and GL primer 2, PCR was performed in a similar procedure and, by introducing the fragment into the KpnI-BglII site of pGL3-basic, HM-700/GL3 containing up to about 700 bp upstream of the transcription initiation point was obtained.

[0102]

Furthermore, with HM-2k/GL3 as the template, using primer HMP700 and 11A' (cagaggattaattaggtaccgaaagagaggtgggctttt) (SEQ ID NO: 21), PCR (98°C for 15 seconds, 65°C for 2 seconds, 74°C for 30 seconds, 25 cycles) was performed using the KOD polymerase (Toyobo) in a Thermal Cycler 480 (Perkin-Elmer, CA, USA). The fragment obtained was inserted into the pCR4 Blunt-TOPO vector using the Zero Blunt TOPO PCR cloning kit for

sequencing ver. B (Invitrogen). The plasmid obtained was treated with a restriction enzyme KpnI, and an about 550 bp fragment was recovered, which was introduced into the KpnI site of HM-125/GL3 using the "ligation high". Thus, dISRE/GL3 lacking -25 to -145 upstream of the transcription initiation point was obtained.

[0103]

## (2) Construction of IRF-2 expression plasmid

The IRF-2 expression plasmid was constructed as follows. From the U266 cells, after eight hours have elapsed after stimulation with interferon- $\alpha$  (1000 U/ml), total RNA was extracted using the TRIzol reagent (GIBCO BRL). With RNA obtained by using the First-strand cDNA Synthesis kit (Pharmacia) as the template, and using NotI-d(T)<sub>18</sub> as the primer, a reverse transcription reaction was performed at 37°C for one hour. With the cDNA obtained as the template, using IRF2-F2 (ttgtattggtagcgtgaaaaaagc) (SEQ ID NO: 22) and IRF2-R2 (cagctagttcacattatctcgtcc) (SEQ ID NO: 23) as primers, PCR (94°C for 45 seconds, 60°C for 45 seconds, 72°C for 60 seconds, 40 cycles) was performed using LA-Taq (Takara Shuzo).

[0104]

With the PCR reaction as the template, using IRF2-F1 (agagggtaccatgccggtggaaaggatgcg) (SEQ ID NO: 24) and IRF2-R1 (agtcggtaccttaactgctcttgacgcggg) (SEQ ID NO: 25) as primers, PCR (94°C for 45 seconds, 60°C for 45 seconds, 72°C for 60 seconds, 30 cycles) was performed using the KOD polymerase (Toyobo). The fragment obtained was treated with a restriction enzyme KpnI, and then introduced into the KpnI site of an expression plasmid pTracer-CMV (Invitrogen) using the ligation high (Toyobo) to obtain a IRF-2 expression plasmid pIRF-2/Tracer.

[0105]

## (3) Measurement of the reporter gene activity

For the introduction of the plasmid into the cells, the polyethyleneimine-Transferrinfection Kit (Tf PEI-Kit) (Bender MedSystems, Vienna, Austria) was used, and for the luciferase



assay the Dual-Luciferase Reporter Assay System (Promega) was used. The cell line was cultured overnight in RPMI-1640 containing 50  $\mu$ M Defferrioxamine and 10% FBS. In order to form a complex of the plasmid to be introduced with Tf-PEI, a mixture of the reporter gene plasmid at a final concentration of 20  $\mu$ g/ml, 20  $\mu$ g/ml of pIRF-2/Tracer or pTracer-CMV, 0.4  $\mu$ g/ml of pRL-SV40, and 1  $\mu$ g/ml of Tf-PEI reagent was prepared and was incubated at room temperature for 20 minutes.

[0106]

5 X 10<sup>5</sup> cells/ml of cells were added at three volumes of the Tf-PEI/plasmid mixture, and was incubated at 37°C for four hours, washed with the medium, and 100  $\mu$ l per well at a concentration of 2 X 10<sup>5</sup> cells/ml was cultured in a 96-well flat-bottomed plate. IFN- $\alpha$  was added to a final concentration of 0 or 1000 U/ml, which was cultured at 37°C for two days. After the cells were washed in PBS(-), it was dissolved in 20  $\mu$ l of the Passive Lysis Buffer, six  $\mu$ l of which was applied to the C96 White Polysorp Fluoronunc plate (Nunc). Using the Luminoskan (Labsystems), luminescence intensity was measured for Firefly and Renilla at 30  $\mu$ l of the substrate at a measurement time of 10 seconds. The measured values were corrected by Firefly/Renilla to obtain the correct relative activity.

[0107]

#### (4) Result

The HM1.24 promoter reporter plasmid and the IRF-2 expression plasmid were introduced into the U266 cells, and the reporter activity was determined (Figure 9). As a result, the luciferase activity was increased in -700 and -151 containing the ISRE motif sequence that is a IRF-2 binding site by IRF-2 co-expression. On the other hand, no changes in activity were noted in dISRE/GL3 that lacks the ISRE sequence by IRF-2 co-expression. This result indicated that IRF-2 binds to the ISRE region of the HM1.24 promoter and enhances

its transcription activity.

[0108]

(5) Confirmation of enhanced expression of HM1.24 antigen by the forced expression of IRF-2

For confirmation of change in an amount of expression of HM1.24, by IRF2, the IRF-2 expression plasmid (pIRF-2/Tracer) or the control plasmid (pTracer/CMV) is introduced into the U266 cells in the method described above, and then cultured for 1-2 days, from which the cells are recovered, and then stained with mouse anti-human HM1.24 antibody as a primary antibody. The cells are washed, and further stained with FITC-labeled anti-mouse IgG antibody as a secondary antibody. After washing the cells, the FITC fluorescence intensity of the cells is measured by flow cytometry. It is confirmed that, in the cells in which the IRF-2 expression plasmid was introduced, there are more cells having a high FITC intensity compared to the cells in which the control plasmid was introduced.

[0109]

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[0111]

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Val	Thr	Asn	Pro	Pro	Asp	Ile	Cys	Gln	Val	Val	Glu	Val	Thr	Thr	Glu
	195						200					205			
Ser	Asp	Glu	Gln	Pro	Val	Ser	Met	Ser	Glu	Leu	Tyr	Pro	Leu	Gln	Ile
	210					215					220				
Ser	Pro	Val	Ser	Ser	Tyr	Ala	Glu	Ser	Glu	Thr	Thr	Asp	Ser	Val	Pro
225					230					235					240
Ser	Asp	Glu	Glu	Ser	Ala	Glu	Gly	Arg	Pro	His	Trp	Arg	Lys	Arg	Asn
				245					250				255		
Ile	Glu	Gly	Lys	Gln	Tyr	Leu	Ser	Asn	Met	Gly	Thr	Arg	Gly	Ser	Tyr
		260						265					270		
Leu	Leu	Pro	Gly	Met	Ala	Ser	Phe	Val	Thr	Ser	Asn	Lys	Pro	Asp	Leu
		275					280					285			
Gln	Val	Thr	Ile	Lys	Glu	Glu	Ser	Asn	Pro	Val	Pro	Tyr	Asn	Ser	Ser
	290						295					300			



Trp Pro Pro Phe Gln Asp Leu Pro Leu Ser Ser Ser Met Thr Pro Ala  
 305 310 315 320

Ser Ser Ser Ser Arg Pro Asp Arg Glu Thr Arg Ala Ser Val Ile Lys  
 325 330 335

Lys Thr Ser Asp Ile Thr Gln Ala Arg Val Lys Ser Cys  
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cytometry using human IgG (control) or anti-HM1.24 antibody as a label.

[Fig. 3]

Figure 3 is a graph showing the result of an experiment in which the U266 cells transformed with a reporter plasmid into which the promoter region of the gene encoding HM1.24 antigen has been inserted were cultured in the absence of interferon- $\alpha$  or in the presence of various concentrations thereof, and then the luciferase activity was determined.

[Fig. 4]

Figure 4 is a graph showing the result of an experiment in which the U266 cells or the HEL cells transformed with a reporter plasmid into which the segment from the transcription initiation point to 151 bp upstream or to 77 bp upstream among the promoter region of the gene encoding HM1.24 antigen has been inserted were cultured in the presence of interferon- $\alpha$ , (1000 U/ml), and then the luciferase activity was determined.

[Fig. 5]

Figure 5 shows the result of an experiment in which a myeloma cell line U266 cultured in the absence (upper) or presence (bottom) of interferon- $\gamma$  was analyzed by flow cytometry using human IgG (control) or anti-HM1.24 antibody as a label.

[Fig. 6]

Figure 6 shows the result of an experiment in which the myeloma cells of the patient cultured in the absence (upper) or presence (bottom) of interferon- $\gamma$  was analyzed by flow cytometry using human IgG (control) or anti-HM1.24 antibody as a label.

[Fig. 7]

Figure 7 is an electrophoretogram that shows changes with time in the amount of transcription factors that are produced by adding IFN- $\alpha$  to the cultured U266 cells and that bind to the HM1.24 promoter region, and is a photograph substituted for drawing. NE(-): no nuclear extract was added. 0 h: the

nuclear extract without IFN- $\alpha$  stimulation was added. 0.5-8 h: the nuclear extract for which the respective time elapsed after stimulation with IFN- $\alpha$  (1000 U/ml) was added. + cold: 50 ng of the nonlabeled ISRE2 probe was added. + cold unrelated: 50 ng of the nonlabeled adp sequence was added. [Fig. 8]

Figure 8 is an electrophoretogram showing the result of an experiment in which the transcription factors that bind to HM1.24 promoter were identified using various antibodies, and is a photograph substituted for drawing. NE(-): no nuclear extract was added. 0 h: the nuclear extract without IFN- $\alpha$  stimulation was added. 8 h: the nuclear extract for which the respective time elapsed after stimulation with IFN- $\alpha$  (1000 U/ml) was added. + cold: 50 ng of the nonlabeled ISRE2 probe was added. + cold unrelated: 50 ng of the nonlabeled adp sequence was added. Two  $\mu$ g each of antibodies was added. [Fig. 9]

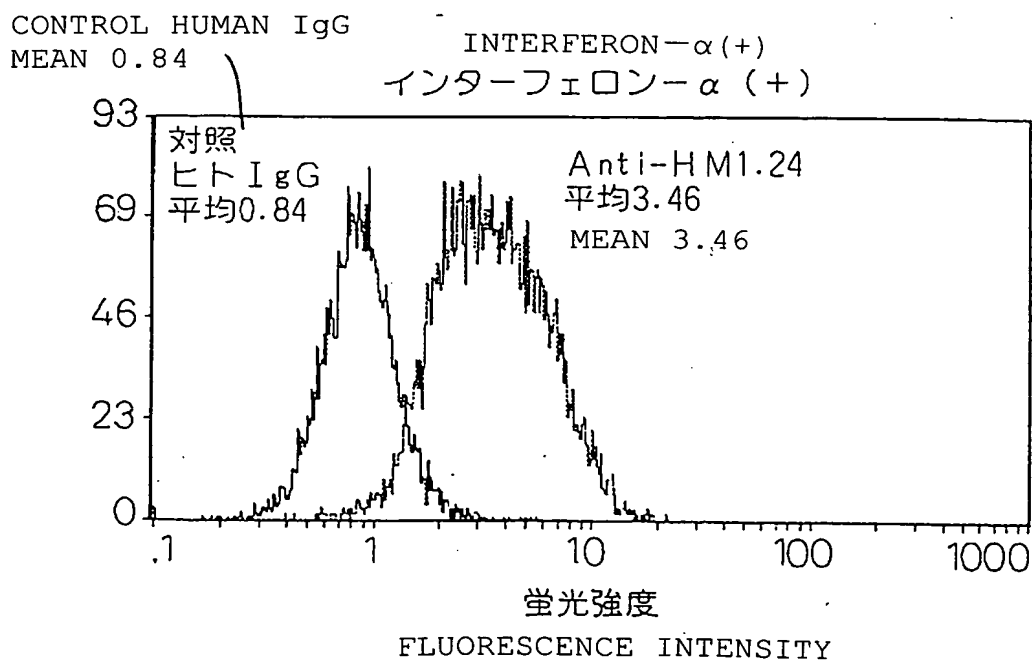
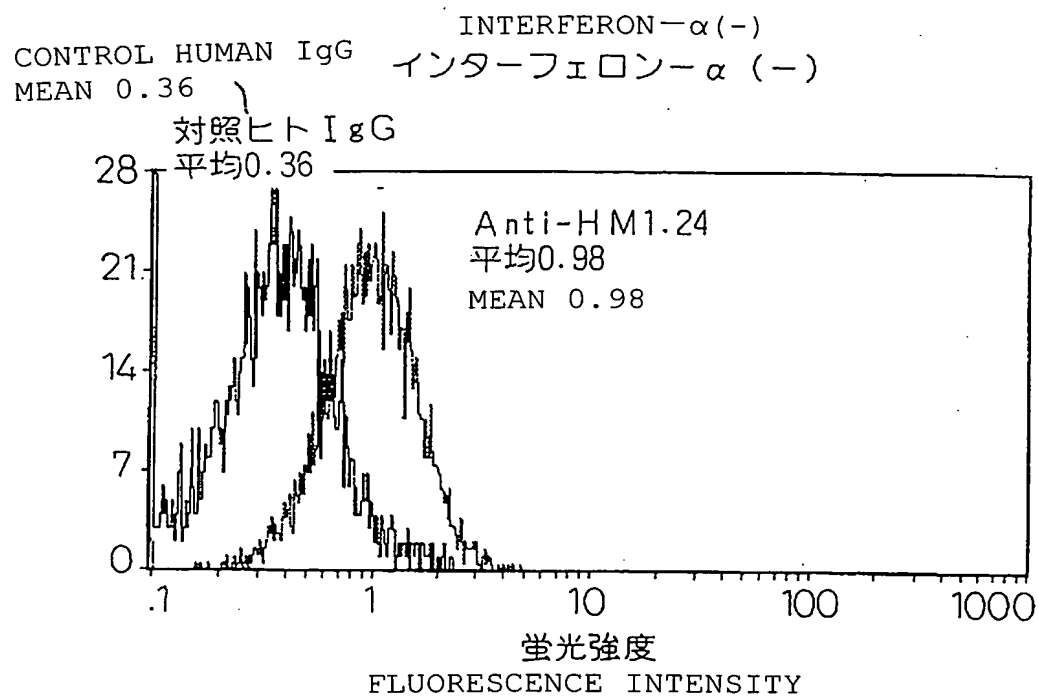
Figure 9 is a graph showing the result of an experiment in which the HM1.24 promoter reporter plasmid and the IRF-2 expression plasmid were introduced into the U266 cells, and the reporter activity was determined.



【書類名】 図面 【NAME OF DOCUMENT】 Drawings

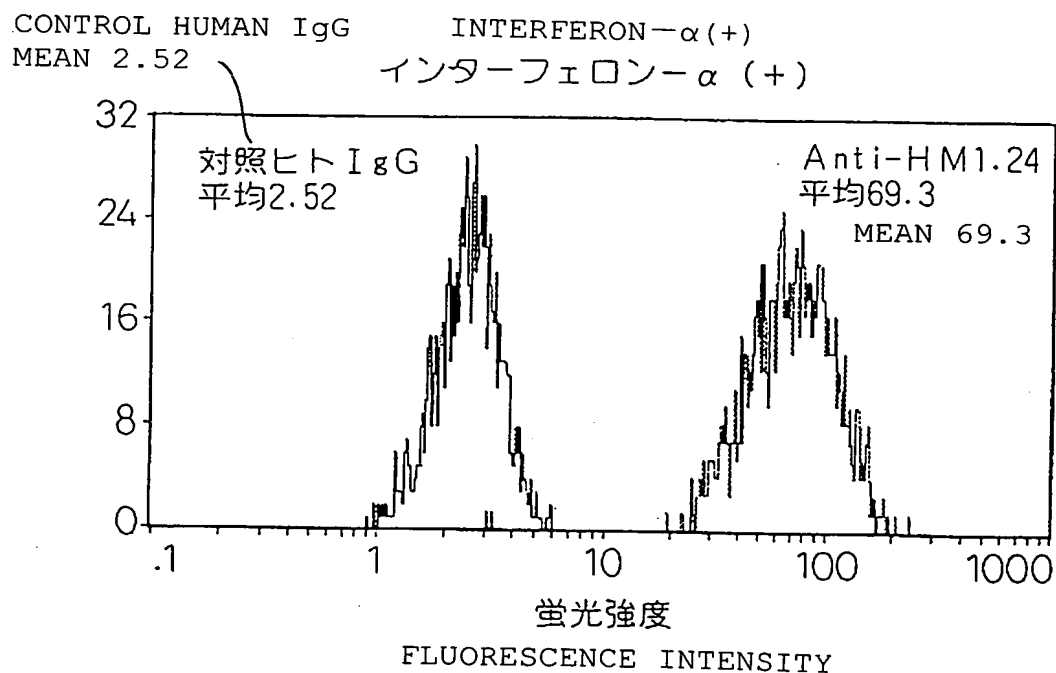
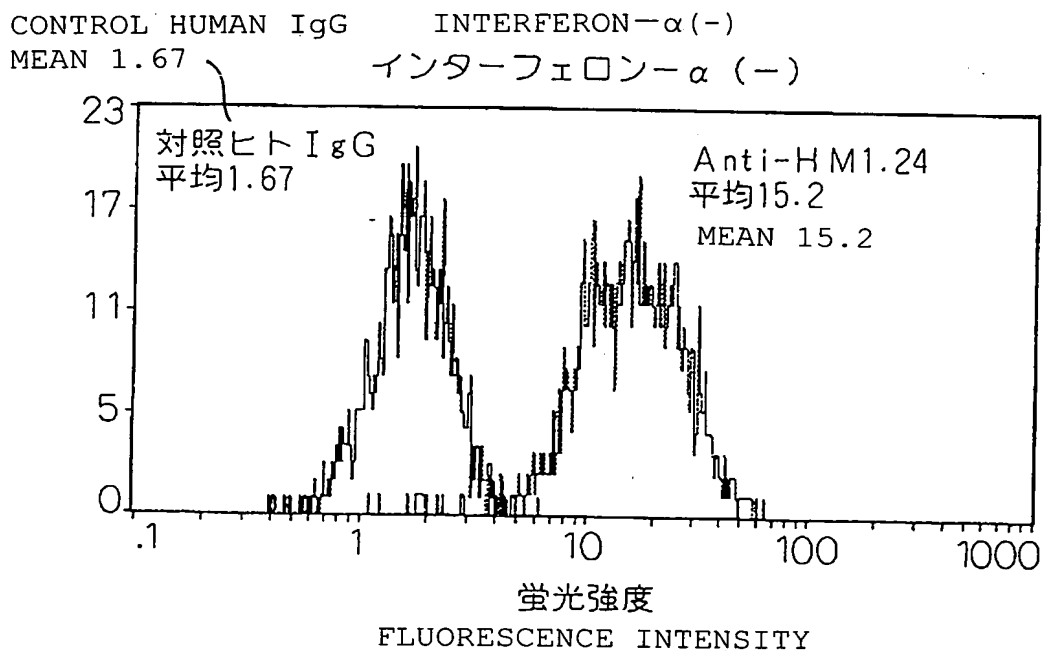
【図 1】 【Fig. 1】

図 1 Fig. 1



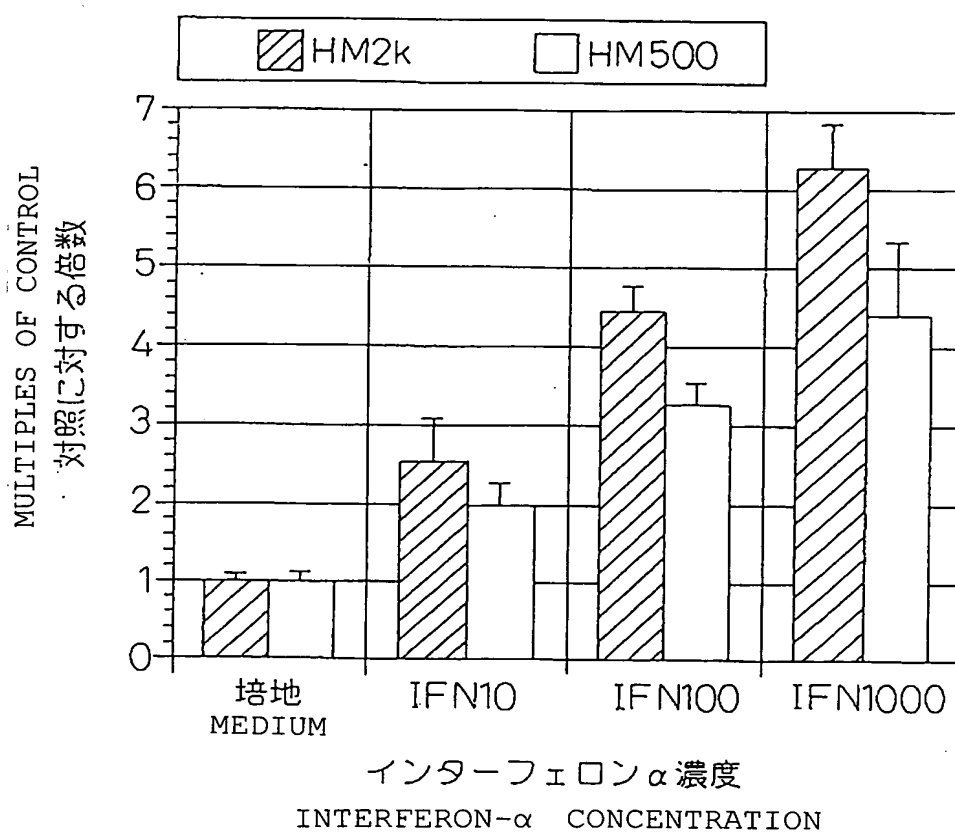
【図2】 【Fig. 2】

図2 Fig. 2



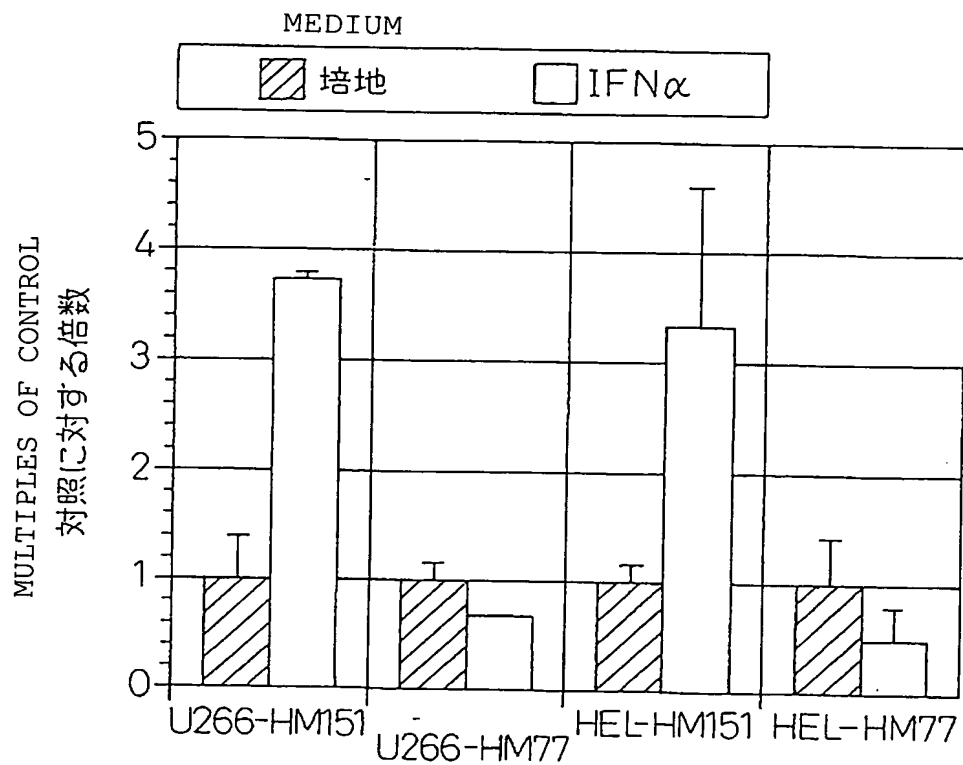
【図3】 【Fig. 3】

図 3 Fig. 3



【図4】 【Fig. 4】

図 4 Fig. 4

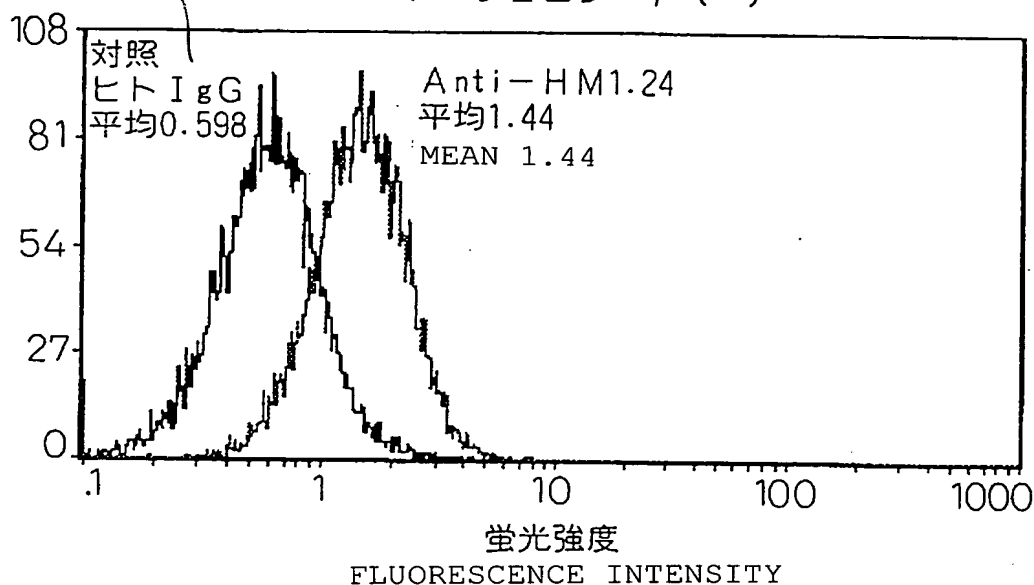


【図5】 【Fig. 5】

図5 Fig. 5

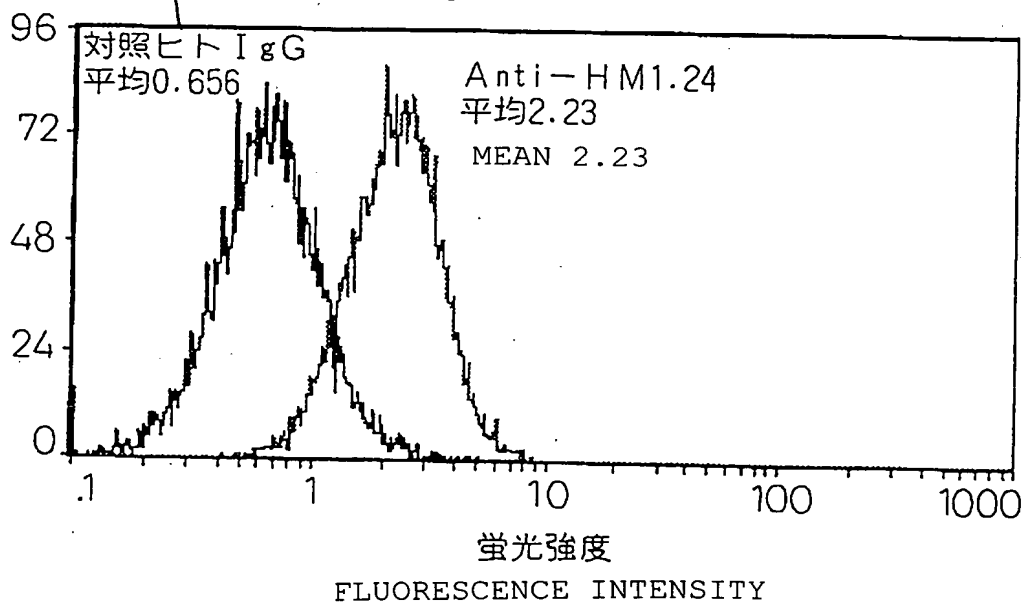
CONTROL HUMAN IgG  
MEAN 0.598

INTERFERON- $\gamma$  (-)  
インターフェロン- $\gamma$  (-)



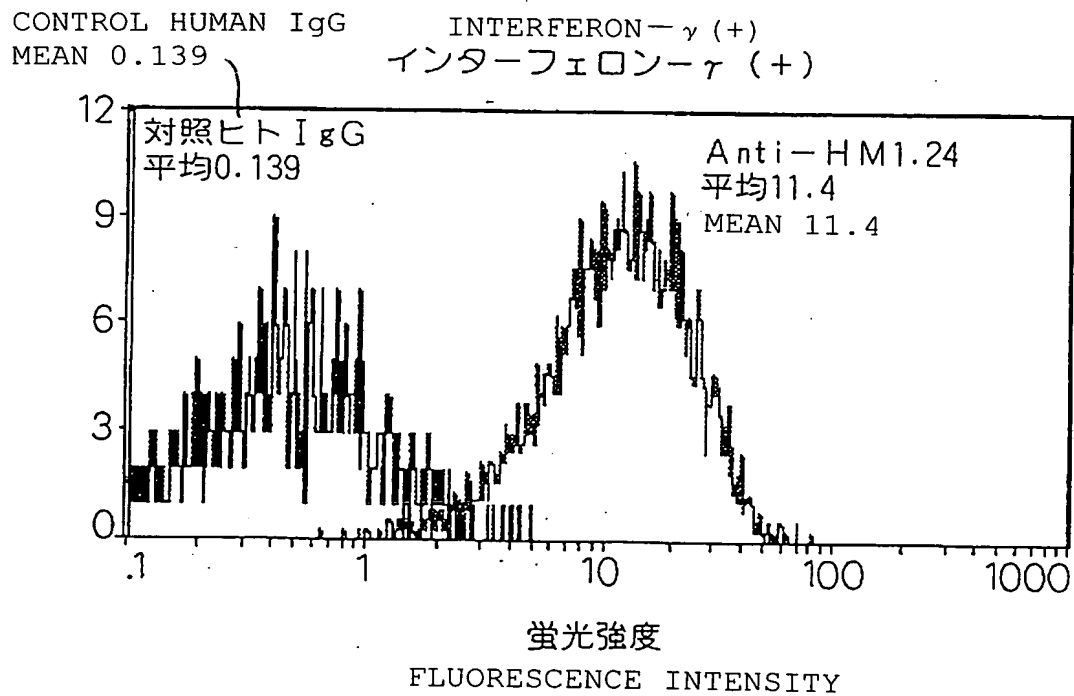
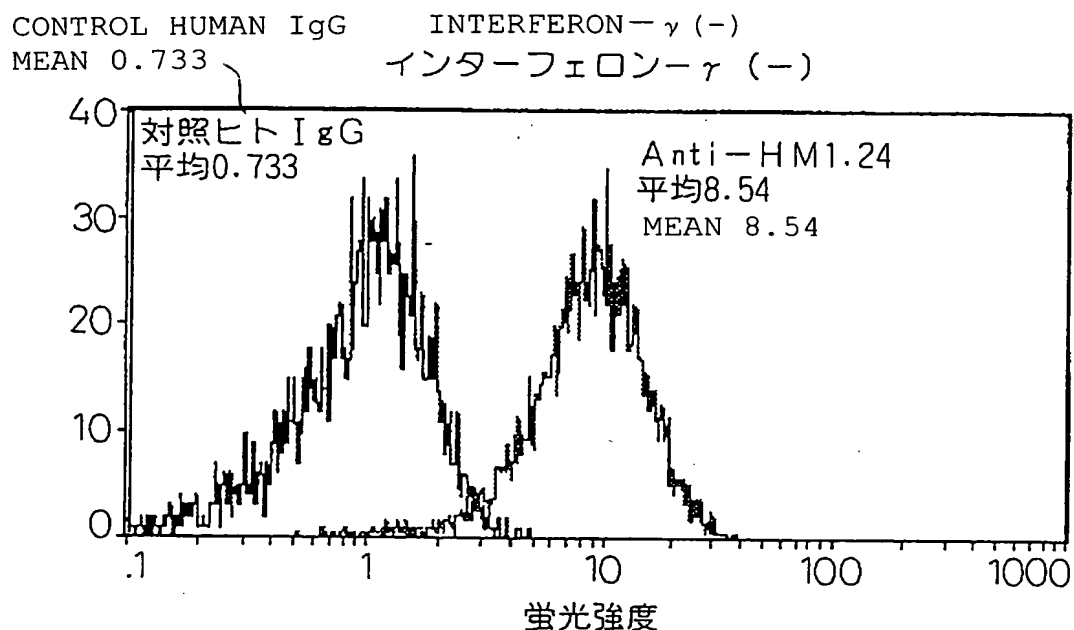
CONTROL HUMAN IgG  
MEAN 0.656

INTERFERON- $\gamma$  (+)  
インターフェロン- $\gamma$  (+)



【図6】 【Fig. 6】

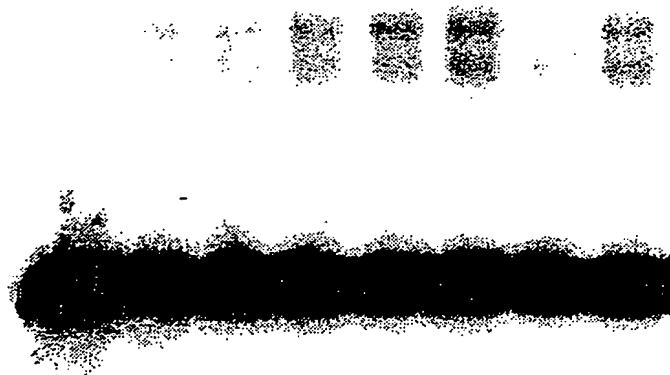
図6 Fig. 6



【図7】【Fig. 7】

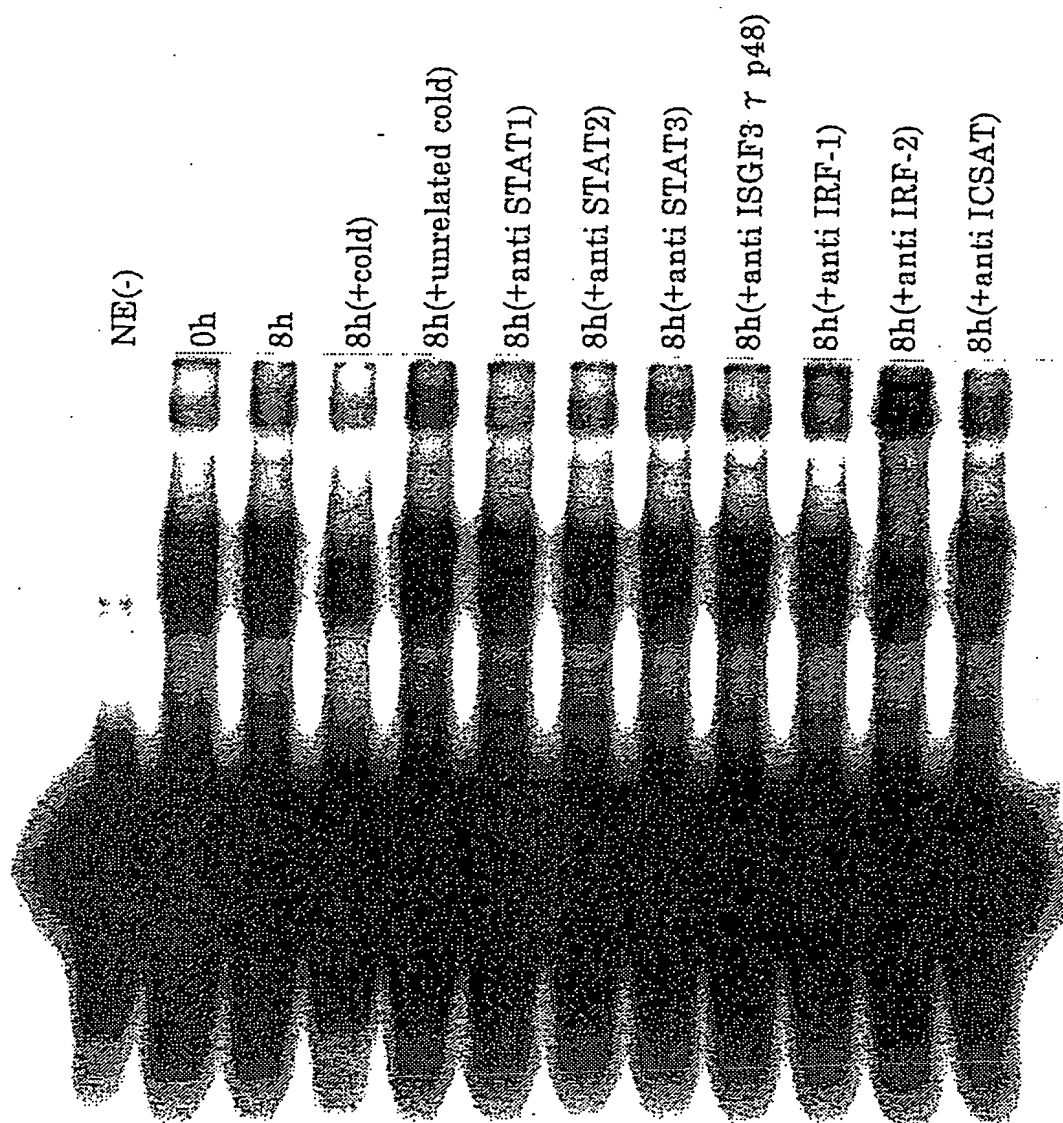
図7 Fig. 7

NE(-)  
0h  
0.5h  
2h  
4h  
8h  
8h(+cold)  
8h(+cold unrelated)



【図8】 【Fig. 8】

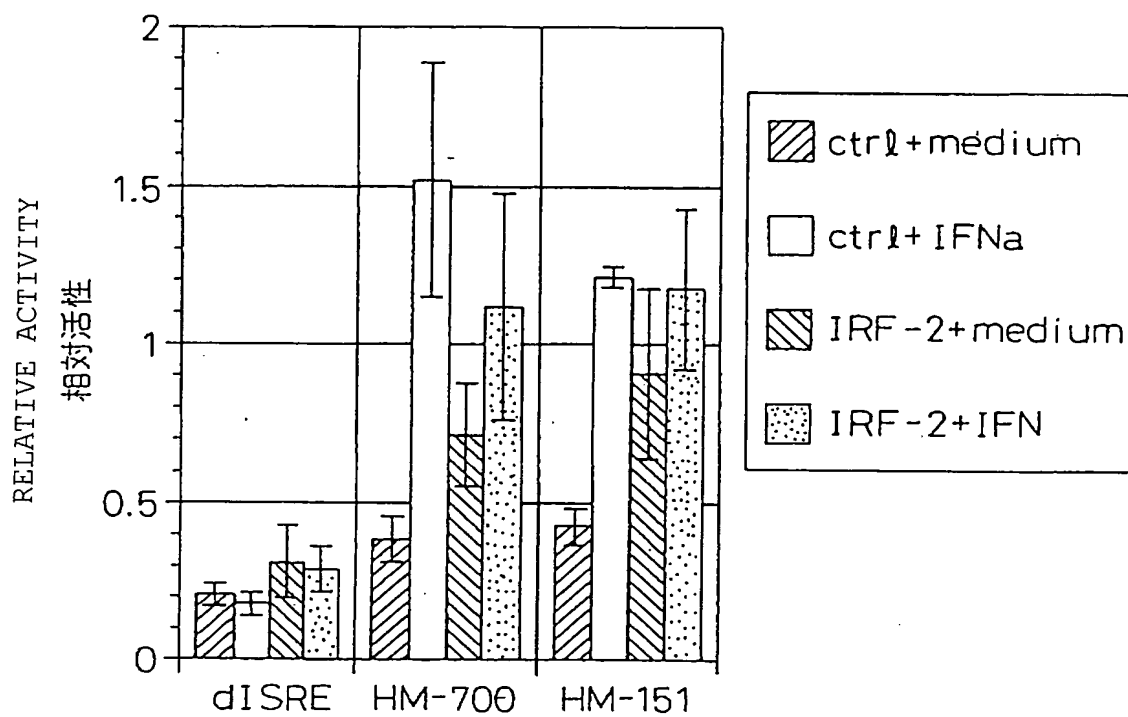
図8 Fig. 8





【図9】 【Fig. 9】

図9 Fig. 9



[NAME OF DOCUMENT] Abstract

[SUMMARY]

[PURPOSE] Provision of a novel mean to enhance the expression of HM1.24 antigen as a target of anti-HM1.24 antigen having anticancer.

[MEANS FOR SOLUTION]

An enhancer of expression, in the myeloma cell, of HM1.24 antigen, said enhancer comprising interferon- $\alpha$  or interferon- $\gamma$ , or IRF-2 protein as an active ingredient. Interferon- $\alpha$  or interferon- $\gamma$  is expected to enhance the expression of HM1.24 antigen by activating the promoter of a gene encoding HM1.24 antigen.

[REPRESENTATIVE DRAWING] None